ICCVAM EXPERT PANEL REVIEW OF IN VITRO TEST METHODS FOR IDENTIFYING OCULAR CORROSIVES AND SEVERE IRRITANTS

Public comment concerning the *"Accuracy and Reliability Reanalysis Addendum"* - Executive Summary and Section IV: HET-CAM Test Method, dated 25 July 2005

# of attachments - 5

by Horst Spielmann (BfR, Berlin, Germany)

My colleagues and I at the BfR, the Federal Institute for Risk Assessment in Berlin, Germany, have in particular reviewed SECTION IV, since we have submitted HET-CAM data for this part of the document. Moreover, I have previously on December 30, 2004, submitted a detailed public comment to the data published in the "HET-CAM Background Review Document (BRD)".
My colleagues and I are pleased that the expert panel and the NICEATM staff has taken most of our comments and recommendations into consideration. However, some of our comments and recommendations, have so far not been evaluated. On behalf of the consortium of more than 12 laboratories that conducted the validation study of the HET-CAM test in Germany and on behalf of the Federal Minister for research and technology, which has funded the study, I want to ask both the members of the expert panel and the staff of NICETAM to take our public comment into consideration before finalizing the BRG and the *"Accuracy and Reliability Reanalysis Addendum* ".

To facilitate the discussion, we are addressing the various points separately.

1. Reference Kalweit et al. 1987, please change to or to Kalweit et al. 1989 or 1990 or to ECVAM-SIS INVITTOX Method #47.

In the executive summary, e.g. in the footnote to Table ES-1, and in the list of references in SECTION IV you are referring to the reference Kalweit et al. 1987. However, in all existing reference databases you will find that the reference Kalweit et al. 1987 does not exist. Dr. Kalweit joined our group in 1988, when the German validation study started.
The correct year for this reference is 1989 as you can see from the attached copy of page 1 of the publication (attachment #1).
In the BRD you have used the correct reference, Kalweit et al. 1989. However, the "Journal of Molecular Toxicology" is not a peer reviewed journal and has only published a single volume in 1989, vol. 1, in which the manuscript was published.
From our perspective it is more appropriate to refer to the first publication in a peer reviewed journal. We therefore want to suggest to use the reference "Kalweit et al., Toxicology in Vitro 4, 720-706, 1990".

2. Correct reference of the IS(B) method

To assess irritation in the HET-CAM test usually the Irritation Score (IS) is used. In the review documents you are referring to two ways of calculating the IS, IS(A) and IS(B):

a) IS(A) is the approach first described by Luepke (1985). IS(A) is calculated from three reactions on the CAM injection (i), haqemorrhage (h) and coagulation.
b) IS(B), which we have used in validation study of the HET-CAM test in Germany, is not taking into account the same endpoints as IS(A) for the following reason:
The endpoint "injection" could not reproducibly be determined in the laboratories participating in the German validation study. Therefore, we have used the three reactions (endpoints) on the CAM haemorrhage (h), lysis of vessels (l) and coagulation (c).

To calculate IS(B), the CAM is observed for a period of 5 min (300 seconds) and the time is recorded, when each reaction is observed on the CAM for the first time. The three reaction times for h, l and c are used to calculate IS(B).

We have first described this method in the publication by Kaltweit et al. 1989. However, for the reasons described above, we suggest to use the reference Kalweit et al. 1990 and the ECVAM-SIS INVITTOX Protocol #47 (IP-47) (attachment #2) , which was first published by our group in 1992 and which is currently available on the internet (http://ecvam-sis.jrc.it/invittox/static/invittox.html). Moreover, we do wonder, why you are not referring to the established INVITTOX Protocol #47 (IP-47) in the BRD and in SECTION IV of the "Addendum".

Recommendation to #1 & #2:
Rather than referring to the reference Kalweit et al. 1987 for the IS(B) method in the executive summary, e.g. in the footnote to Table ES-1, and in the list of references of SECTION IV, I want to ask you to use the correct references, which underwent a peer review process as described above Kalweit et al. 1990 and ECVAM-SIS INVITOX Protocol #47 (IP-47) (http://ecvam-sis.jrc.it/invittox/static/invittox.html).

3. Differences between IS(A) and IS(B) methods for calculating the irritation potential in the HET-CAM test

As outlined above, Luepke (1985) used "injection" as one of the three endpoints for determining IS(A) to assess the irritaiton potential on the CAM, while we used "lysis of vessels" (l) instead to calculate IS(B). Therfore, the information provided by IS(A) and IS(B) is not identical. Moreover, the algorithms for calculating IS(A) and IS(B) are not taking into account identical time periods for determining the first appearance of the three reactions (endpoints) on the CAM.
Thus, the experts of NICEATM and of the ICCVAM Expert Panel have to ensure from the "materials and methods" section of each HET-CAM study, which of the prediction models IS(A) and IS(B) has been applied and, consequently take this information into account when evaluating the *"Accuracy and Reliability"* for each of the HET-CAM studies.

Recommendation #3:
Since it is not clear from the executive summary and from SECTION IV "HET-CAM test" that the specific differences between IS(A) and IS(B) have been taken into account in the evaluation by the NICETAM team, this important detail of the HET-CAM test should be reevaluated before the report is finalized.

4. Evaluation of the classification of test chemicals in the HET-CAM test
Both in the ECVAM-SIS INVITTOX Protocol #47 (IP-47) and in our publications Spielmann et al. 1993 and 1996, we have described the prediction model that was used in the German validation study of the HET-CAM test, to classify test chemicals according to their eye irritation potential on the eye. This prediction model takes into account both the IS(B) and the "threshold concentration" (TH). Thus, in the German validation study of the HET-CAM test, the IS(B) was never used as prediction model for classifying severely irritating test materials.

In their evaluation given in the *"Accuracy and Reliability Reanalysis Addendum: HET-CAM Test Method - SECTION IV"* the authors do not explain, why they have only used the IS(B) for classifying chemicals tested in the German HET-CAM test validation study and not the prediction model used in the German validation study as described both in the ECVAM-SIS INVITTOX method #47 and in the publications by the German consortium (Spielmann et al. 1993 and 1996):

Recommendation #4:
It must be discussed in the *"Accuracy and Reliability Reanalysis Addendum"*, why the authors did not consider to use the prediction model used in the German validation study of the HET-CAM test, which is also the current prediction model in the ECVAM-SIS INVITTOX Protocol #47 (IP-47).

5. Correction to Table IV-8 in the Addendum to the In Vitro ocular Toxicity Draft BRD
The content of Table VI-8 is discussed in Section IV on pg. IV-25-IV-27 under the heading *"2.4 Accuracy of the HET-CAM IS(B) Analysis Method for the GHS Ocular Hazard Classification System by Chemical Class and Property of Interest"*. Taking into account the number of chemicals evaluated in Table IV-8, we do assume that the data were taken from pour publications Spielmann et al., 1993 and 1996. However, the authors are not giving reference to any publication in chapter 2.4.
Moreover, since we have tested chemicals both in 10% and 100% solutions and since the numbers of chemicals analyzed is in the range of the numbers of chemicals tested in the German validation study, we do assume that the classifications are based on our results.

As outlined above, the prediction model for classifying test chemicals in the German validation study is not based on the IS(B), which was used by the NICEATM. The BGA prediction model for classifying test chemicals according to their eye irritation potential Table II on pg. 761 of our publication Spielmann et al. ATLA 24, 741-858, 1996. The classification results obtained in the German validation study of the HET-CAM test are given in Table III on the same pg. 761 of the same publication. The authors of the *"Accuracy and Reliability Reanalysis Addendum"* do not report our results and they do not discuss them in comparison to their classification results.
From the scientific point of view, we find it unacceptable that this point is not discussed throughout the *"Accuracy and Reliability Reanalysis Addendum".*

Moreover, we are amazed about the classification results given on pg. IV-26 and IV-27. On pg. IV-26 the following results are given for false positive and false negative rates: For the 101 "overall IS(B)-10" chemicals and the 143 "over all IS(B)-110" chemicals false positive rates of 33 and 60% are given and false negative rates of 30% and 15% respectively. This

seems to be in contrast to the data given in the same table on pg. IV-27, since for 40 chemicals of *"category 1 subgroup IS(B)-10"* a false positive rate of 0% is reported and for 37 chemicals of *"category 1 subgroup IS(B)-100"* again the false positive rate is 0%.

Recommendation #5:
Since chapter 2.4 in SECTION IV is not meeting the scientific standard of a peer reviewed publication it has to be redrafted before it can be recommended for publication.

6. Reproducibility of classification results in the HET-CAM test
The first stage of the validation study of the HET-CAM test was conducted as an inter-laboratory validation study. The first short publication of this part of the study is entitled "Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany" (Spielmann et al, Toxic. In Vitro 5, 539-542, 1991) (attachment #4). Table 2 of this publication is entitled "A comparison of the results of the HET-CAM test carried out in 12 laboratories and the in vivo irritation potential (as assessed in the Draize eye test) for 27 chemicals". For your information I am attaching a copy of this publication.
Even when taking into account that the classification in the Draize eye test has slightly been changed according to harmonization of OECD Test Guidelines, the classification results given in table 2 for severely irritating an corrosive chemicals is impressive. According to the IS(B) scoring system a score >10 indicates severe eye irritation properties. According to the footnote to table 2, a substance was classified positive, when 75% of laboratories determined an IS(B) of >10. As you can see all of the corrosive chemicals were classified correctly and the majority of severely irritating test materials were also correctly classified.
This result is the main reason that regulators in Europe are accepting a positive results in the HET-CAM test for the classification of corrosive and severely irritating materials without any confirmatory testing in rabbits in vivo.
This important information has not been covered in the *"Accuracy and Reliability Reanalysis Addendum".* Sine it is the goal of the joint ICCVAM/ECVAM exercise of reviewing existing data obtained in the four established in vitro alternatives to the Draize eye test, I have to ask the authors of the study and also the ICCVAM Expert Panel to comment on this important piece of evidence.

Recommendation #6:
It must be discussed in the *"Accuracy and Reliability Reanalysis Addendum"*, why the authors did not report and discuss the reproducibility of the classification results for test chemicals, which are corrosive or severely irritating to the eye. In addition the consequences for testing these materials in vivo in rabbits should be explained.

7. References Spielmann H, Liebsch M 2005a and 2005b on pg. IV-88
The two references, which are given in Section IV of the *"Accuracy and Reliability Reanalysis Addendum"*, are not correct for the following reasons:
We have in June of 2004 submitted to ICCVAM/NICETAM the publication in which the validation study of the HET-CAM test in Germany is described in detail (Spielmann et al. ATLA 24, 741-858, 1996) and we have also sent the background material as an "xls-file" to the scientists, who were responsible for the HET-CAM test at NICEATM. For your information I am attaching a copy of the e-mail, in which Dr. Neepa Choksi on July 8, 2004, confirmed that

she had received the electronic version of the German validation study. Attached to that e-mail is my e-mail dated June 14, 2004, in which I had sent the "xls-file" to Drs. Choksi, Stokes and Tice (attachment #4).
Thus, Dr. Manfred Liebsch and I did not submit "Unpublished data provided directly to NICEATM by H.Spielmann and M. Liebsch", as quoted in the references Spielmann H, Liebsch M 2005a and 2005b on pg IV-88.

Recommendation #7:
For the reasons explained, Dr. Manfred Liebsch and I would appreciate if the quotation in the two references is changed to "Unpublished data provided directly to NICEATM by H.Spielmann and M. Liebsch".

8. Publication missing in the literature provided with the BRDs and the Addendum
In my public comment dated December 30, 2004, to the BRD (attachment #5) I had clearly addressed that an important publication on the evaluation of different protocols of the HET-CAM test that was published under the auspices of IRAG, the Interagency Regulatory Alternatives Group, the predecessor of ICCVAM. My colleagues and I wonder, why this important publication has not been evaluated in the "Addendum SECTION IV". I am, therefore, repeating my request by copying the section from my earlier public comment to the BRD:
"IRAG Working group 2 CAM-based assays" by Spielmann et al., 1997, Food and Chemical Toxicology 35, 39-66.
In 1993 the US Interagency Regulatory Alternatives Group (IRAG) held a workshop on “Eye irritation testing; practical applications of non-whole animal alternatives”. For several in vitro alternatives, which are currently evaluated by the ICCVAM expert panel review, extensive analysis of the in vivo/in vitro correlations have been assessed. I wonder why the NICEATM expert group did not provide the expert reviewers panel with these documents but only mentioned them in the list of references. The one to which I have contributed may be helpful for the experts working on the HET-CEM BRD. Moreover, this activity was sponsored by several of the Federal US agencies, which are stakeholders of ICCVAM today.

Recommendation #8:
This publication should be added to the official list of references provided and it must be evaluated and commented on in SECTION IV of the "Addendum".